



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

For: Charge Reversal of Polyion Complexes

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.131

Dear Sir:

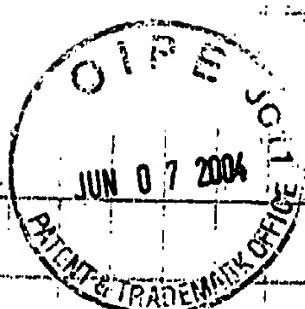
I, an inventor, Vladimir S. Trubetskoy, hereby declare as follows:

1. I am an inventor of the captioned application.
2. Photocopies of pages from my, Vladimir Trubetskoy's, personal laboratory notebook showing recharging of DNA/polycation particles beginning on December 16, 1997 accompany this Declaration.
3. It is known to me that the process performed in the notebook pages results in the formation of negatively charged tertiary complexes as described in the present specification.
4. The recharging process was conceived prior to the effective date of the Office Action prior art reference.
5. Developed of the recharging process occurred with due diligence from conception to the filing of the application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity ~~of~~ the application or any patent issued thereon.

Vladimir S. Trubetskoy Date

6/7/04



1.5 ml of react mix applied to
Aldrich preparative TLC silica
plate and run in
CHCl₃/MeOH (65:20) system
product

Product band was scraped off the
plate.

16/97

Work with STDNA-be's mS.

Part of silica (above) was washed with

① CHCl₃

② CHCl₃/MeOH 65:20

Substantial amounts of
SSS (upper spot is present)

Whole amount of silica was washed with

① CHCl₃/MeOH 65:10

② G 11 65:30 → this fraction
was evaporated

Work on recharging surface of caged DNA particles.

Caged particles are positively charged. If you add
excess of polyanion it can recharge the surface
to the opposite charge.

Caged particles were prepared in Buelke's
conditions (p. 72)

After 2h of incubation of each mix at room to

The mixture was diluted twice with deionized H_2O and to 12 μg DNA/48 μg PLL caged, 500 μg of polymethacrylic acid (pMAA) were added.

No. FI Conc.

| | | |
|---|---------|------------------------------------|
| 1 | 239.385 | -10408 DNA/PLL (1:6) caged 1.7DTBP |
| 2 | 525.217 | -22835 +500 μg pMAA |
| 3 | 392.396 | -17060 after centrifug. |
| 4 | 720.091 | -31308 +150 μg NaCl |
| 5 | 481.248 | -20923 after centrifug. |

ζ -potential was also measured

| Run | Zeta Potential (mV) | Half Width (mV) |
|------------|---------------------|-----------------|
| 1 | 7.66 | 2.34 |
| 2 | 8.01 | 2.18 |
| 3 | 8.08 | 2.22 |
| 4 | 10.20 | 2.58 |
| 5 | 8.06 | 2.63 |
| 6 | 6.74 | 2.25 |
| 7 | 6.69 | 2.29 |
| 8 | 23.20 | 2.26 |
| 9 | 8.05 | 2.24 |
| 10 | 27.46 | 4.86 |
| Mean | 11.41 | 2.58 |
| Std. Error | 2.36 | 0.26 |

PLL/DTBP(6,1.7)nosalt (Run 10)

VT

,DNA=17 $\mu\text{g}/\text{ml}$, 17 mM HEPES, pH 8.0

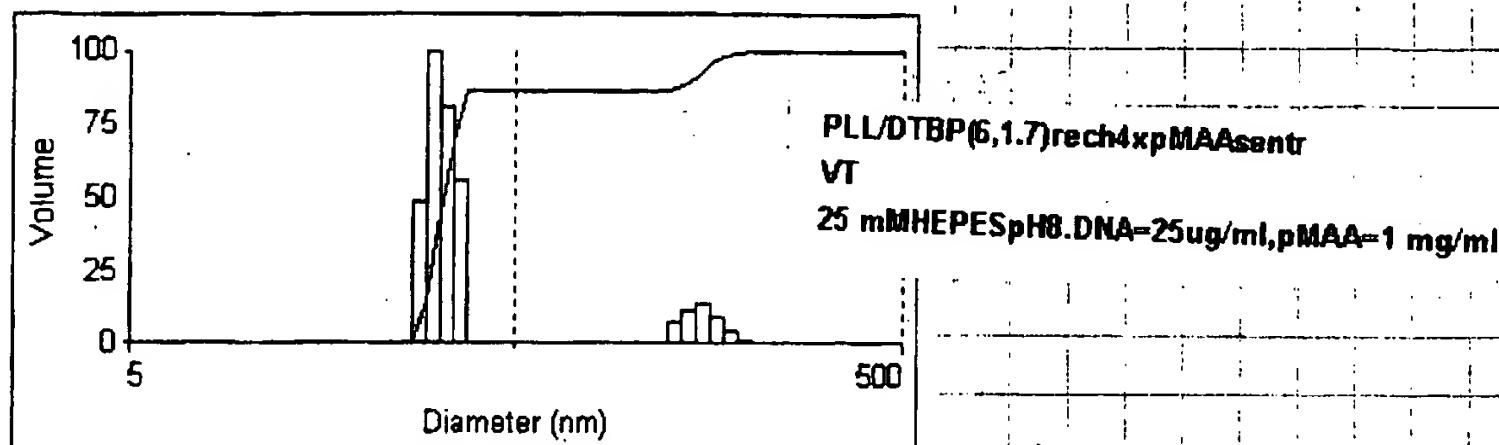
| Run | Zeta Potential (mV) | Half Width (mV) |
|------------|---------------------|-----------------|
| 1 | -29.03 | 2.80 |
| 2 | -7.70 | 4.06 |
| 3 | -15.37 | 2.74 |
| 4 | -25.43 | 3.53 |
| 5 | -63.89 | 2.89 |
| 6 | -16.53 | 2.89 |
| 7 | -28.26 | 2.63 |
| 8 | -24.13 | 3.00 |
| 9 | -26.00 | 7.24 |
| 10 | -35.16 | 4.16 |
| Mean | -26.15 | 3.59 |
| Std. Error | 3.97 | 0.44 |

PLL/DTBP(6,1.7)+4xpMAAnosalt (Run 10)

VT

,DNA=17 $\mu\text{g}/\text{ml}$, 17 mM HEPES, pH 8.0

After addition of pMAA, I_{20} is increasing somewhat but still particle size is



30.20/114.1g/150

Basically the same effect was observed with dextran-sulfate(DS) as counterion.

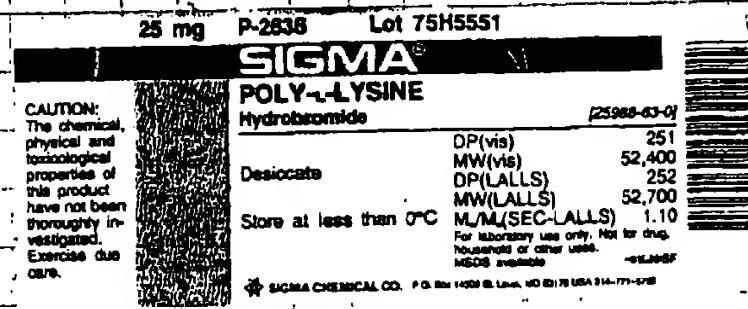
the mixture was as indicated on p 25 with exception that DS was added instead of pMAA

| Run | Zeta Potential (mV) | Half Width (mV) |
|------------|---------------------|-----------------|
| 1 | 33.22 | 2.41 |
| 2 | 27.98 | 2.61 |
| 3 | 20.17 | 3.26 |
| 4 | 26.99 | 2.22 |
| 5 | 10.37 | 2.35 |
| 6 | 27.01 | 2.06 |
| 7 | 33.33 | 2.24 |
| 8 | 26.83 | 4.46 |
| 9 | 28.83 | 2.93 |
| 10 | 29.39 | 2.18 |
| Mean | 26.31 | 2.67 |
| Std. Error | 2.13 | 0.23 |

PLL/DTBP(6,1.7)nosalt (Run 10)
VT
,DNA=25ug/ml, 25 mM HEPES, pH 8.0, DS=0.5mg/ml

| Run | Zeta Potential (mV) | Half Width (mV) |
|------------|---------------------|-----------------|
| 1 | -7.34 | 2.32 |
| 2 | -22.67 | 2.92 |
| 3 | -13.63 | 2.19 |
| 4 | -15.95 | 6.66 |
| 5 | -2.65 | 3.97 |
| 6 | -21.18 | 2.29 |
| 7 | -25.78 | 2.10 |
| 8 | -13.92 | 2.42 |
| 9 | -11.06 | 2.01 |
| 10 | -15.94 | 6.32 |
| Mean | -15.00 | 3.21 |
| Std. Error | 2.23 | 0.50 |

PLL/DTBP(6,1.7)+500ugDSnosalt (Run 10)
VT
,DNA=25ug/ml, 25 mM HEPES, pH 8.0, DS=0.5mg/ml



12/17/97 Titration of DNA/PLL (1:6) caged and non-caged with dextran sulfate.

Buckler's solution was prepared as described

in p. 72 this volume.

V = 1.5 ml (30 g DNA / 114 g PLL)

50 g - 500 g of dextran sulfate (Mr = 500 kDa, Sigma)

were added to each sample and

TOTO, size and ζ -potentiol were

measured. Some non-caged samples were measured

in the same conditions

TOTO concentrations: (8 λ of stock TOTO into 20 μ l of

25 mM HEPES, pH 8.0; 10 λ of sample \rightarrow 0.5 μ l TOTO)

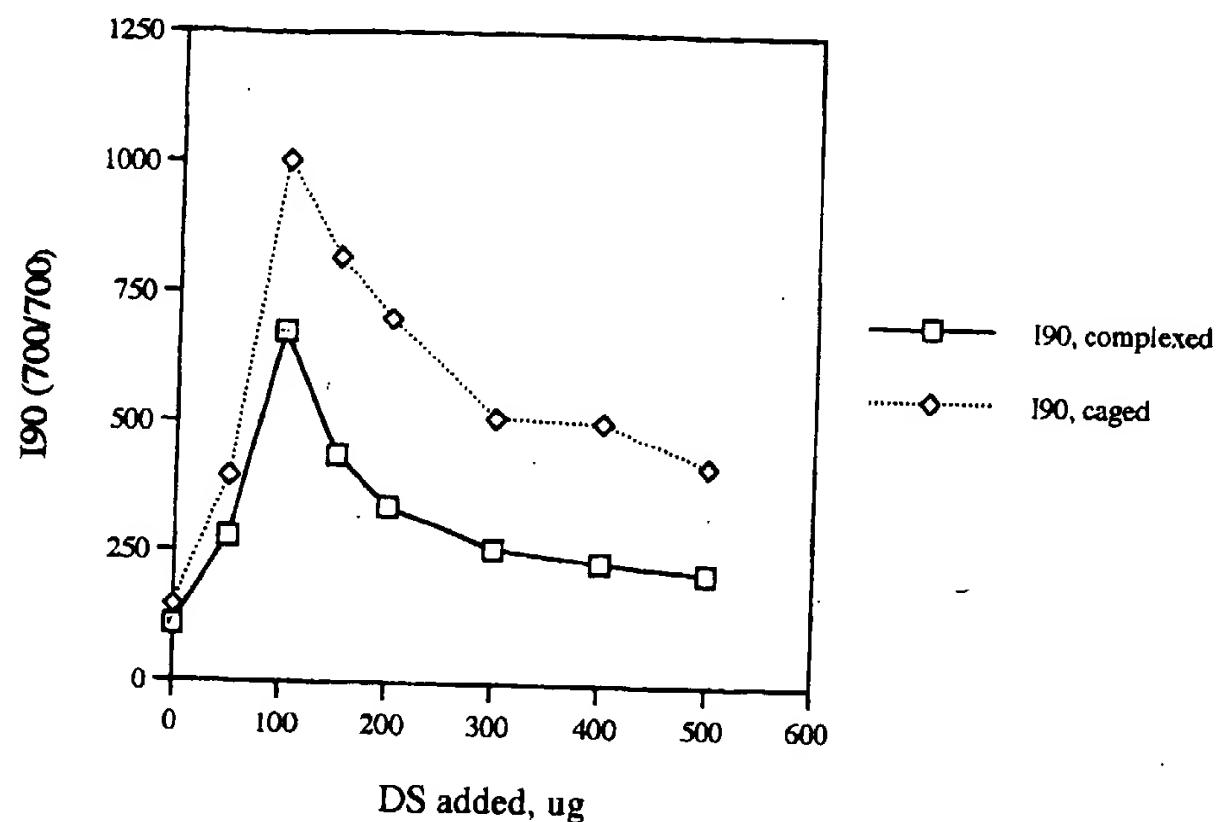
Complexed

| No. | Caged | FI | Conc. |
|-----|----------|---------|-------|
| 1 | 145.344 | -6319.3 | 0 |
| 2 | 395.046 | -17175 | 50 |
| 3 | 1004.619 | -43679 | 100 |
| 4 | 819.067 | -35611 | 150 |
| 5 | 702.273 | -30533 | 200 |
| 6 | 512.809 | -22296 | 300 |
| 7 | 504.484 | -21934 | 400 |
| 8 | 421.555 | -18328 | 500 |

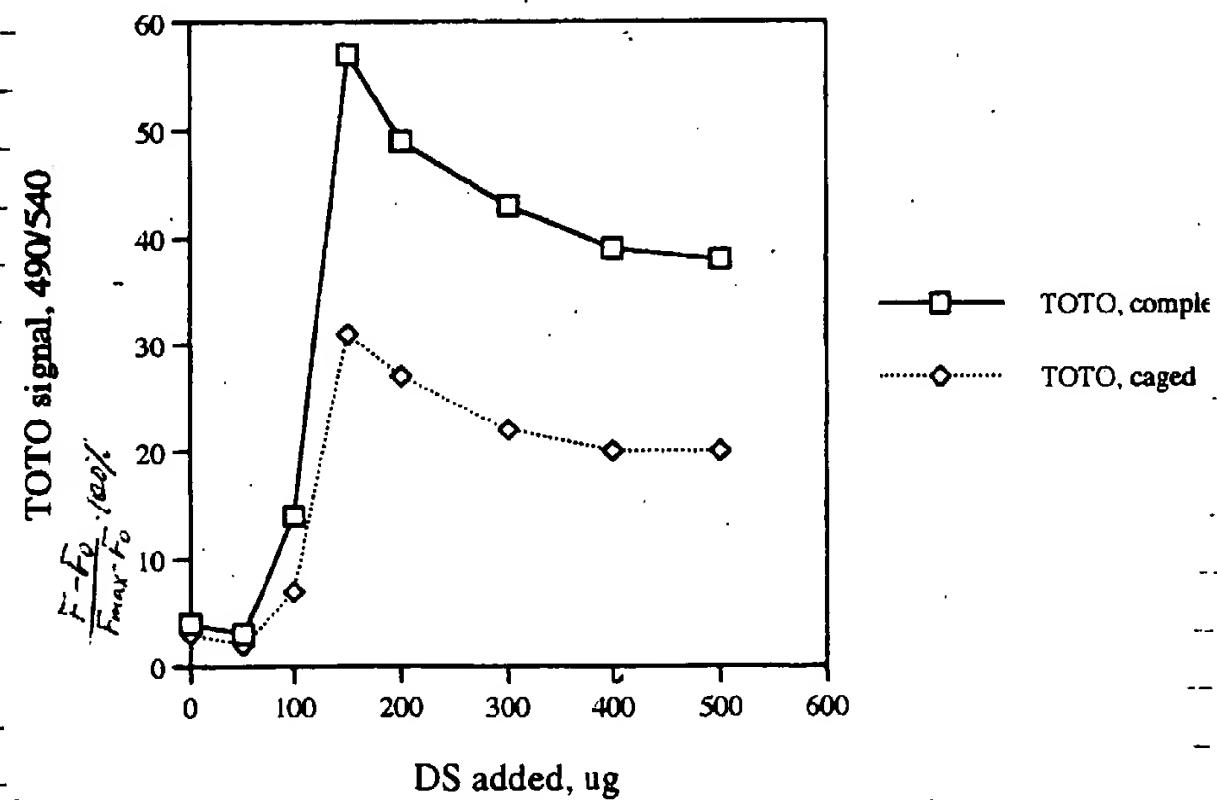
| No. | FI | Conc. |
|-----|---------|---------|
| 1 | 28.999 | -1260.8 |
| 2 | 687.116 | -29874 |
| 3 | 47.693 | -2073.6 |
| 4 | 38.309 | -1665.6 |
| 5 | 72.144 | -3136.7 |
| 6 | 234.264 | -10185 |
| 7 | 203.611 | -8852.7 |
| 8 | 175.301 | -7621.8 |
| 9 | 161.145 | -7006.3 |
| 10 | 160.371 | -6972.7 |

| No. | FI | Conc. |
|-----|---------|---------|
| 1 | 108.628 | -4723.0 |
| 2 | 278.651 | -12115 |
| 3 | 676.371 | -29407 |
| 4 | 435.570 | -18937 |
| 5 | 338.690 | -14725 |
| 6 | 258.092 | -11221 |
| 7 | 234.890 | -10212 |
| 8 | 215.716 | -9379.0 |
| No. | FI | Conc. |
| 1 | 96.057 | -1170.4 |
| 2 | 533.456 | -23193 |
| 3 | 64.342 | -2797.5 |
| 4 | 60.599 | -2634.7 |
| 5 | 111.724 | -4857.6 |
| 6 | 322.742 | -14032 |
| 7 | 284.332 | -12362 |
| 8 | 253.480 | -11020 |
| 9 | 236.314 | -10274 |
| 10 | 230.641 | -10027 |
| 11 | 43.587 | -1895.1 |

Stabilization of DNA/PLL complexes (caged and complexed) with dextran sulfate



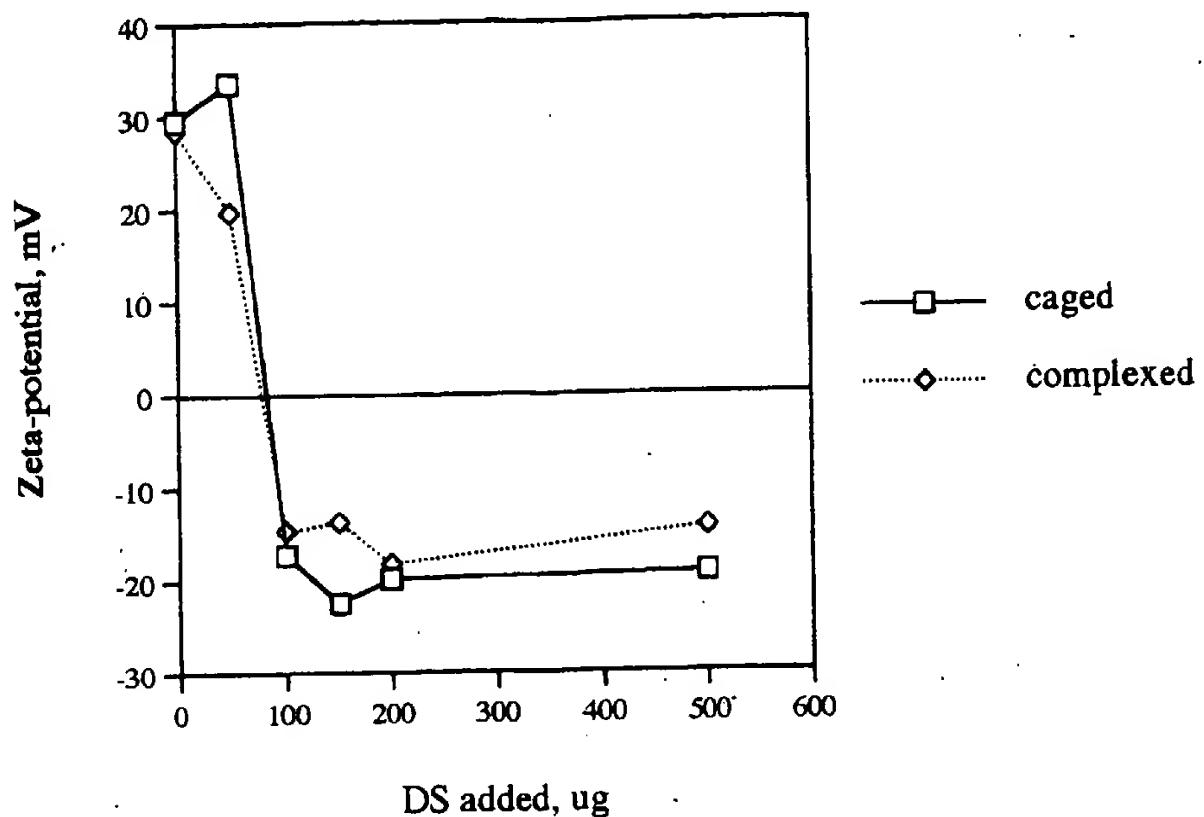
Condensation of DNA/PLL/DS complexes (caged and complexed)



Complexed DNA/PLL were prepared in the same conditions as for caged but w/o γ -linking with DTTBP.

ζ -potential is changed to opposite at 100 ug DS added.

Zeta-potential of DNA/PLL/DS complexes, no salt



| Run | Zeta Potential (mV) | Half Width (mV) |
|------------|---------------------|-----------------|
| 1 | 31.34 | 3.67 |
| 2 | 33.02 | 2.11 |
| 3 | 26.96 | 3.57 |
| 4 | 39.37 | 1.96 |
| 5 | 30.17 | 2.31 |
| 6 | 24.25 | 2.10 |
| 7 | 26.53 | 1.95 |
| 8 | 22.45 | 2.10 |
| 9 | 29.20 | 1.85 |
| 10 | 29.55 | 2.96 |
| Mean | 29.28 | 2.46 |
| Std. Error | 1.51 | 0.22 |

| Run | Zeta Potential (mV) | Half Width (mV) |
|------------|---------------------|-----------------|
| 1 | 49.36 | 3.06 |
| 2 | 44.33 | 1.83 |
| 3 | 37.00 | 1.80 |
| 4 | 33.83 | 3.36 |
| 5 | 39.11 | 2.34 |
| 6 | 27.81 | 1.81 |
| 7 | 28.67 | 4.53 |
| 8 | 11.79 | 1.82 |
| 9 | 36.92 | 1.84 |
| 10 | 28.00 | 3.19 |
| Mean | 33.68 | 2.56 |
| Std. Error | 3.30 | 0.30 |

| Run | Zeta Potential (mV) | Half Width (mV) |
|------------|---------------------|-----------------|
| 1 | -18.29 | 1.86 |
| 2 | -8.36 | 1.87 |
| 3 | -6.31 | 1.93 |
| 4 | -14.62 | 1.93 |
| 5 | -14.66 | 1.89 |
| 6 | -21.63 | 1.83 |
| 7 | -18.70 | 1.81 |
| 8 | -25.67 | 2.50 |
| 9 | -22.83 | 2.45 |
| 10 | -21.59 | 2.07 |
| Mean | -17.26 | 2.01 |
| Std. Error | 1.98 | 0.08 |

| | | | |
|------------|-------|------|-----------------------------------|
| 4 | 39.37 | 1.96 | PLL/DTBP(6,1.7) (Run 10) |
| 5 | 30.17 | 2.31 | VT |
| 6 | 24.26 | 2.10 | |
| 7 | 28.53 | 1.96 | ,DNA=20ug/ml, 25 mM HEPES, pH 8.0 |
| 8 | 22.45 | 2.10 | |
| 9 | 29.20 | 1.85 | |
| 10 | 29.55 | 2.96 | |
| Mean | 29.28 | 2.46 | |
| Std. Error | 1.61 | 0.22 | |

| Run | Zeta Potential (mV) | Half Width (mV) | |
|------------|---------------------|-----------------|-----------------------------------|
| 1 | 49.36 | 3.06 | |
| 2 | 44.33 | 1.83 | |
| 3 | 37.00 | 1.80 | |
| 4 | 33.83 | 3.36 | PLL/DTBP(6,1.7)+50ugDS (Run 10) |
| 5 | 39.11 | 2.34 | VT |
| 6 | 27.81 | 1.81 | |
| 7 | 28.67 | 4.53 | ,DNA=20ug/ml, 25 mM HEPES, pH 8.0 |
| 8 | 11.79 | 1.82 | |
| 9 | 36.92 | 1.84 | |
| 10 | 28.00 | 3.19 | |
| Mean | 33.68 | 2.66 | |
| Std. Error | 3.30 | 0.30 | |

| Run | Zeta Potential (mV) | Half Width (mV) | |
|------------|---------------------|-----------------|-----------------------------------|
| 1 | -18.29 | 1.86 | |
| 2 | -8.36 | 1.87 | |
| 3 | -6.31 | 1.93 | |
| 4 | -14.62 | 1.93 | |
| 5 | -14.56 | 1.89 | PLL/DTBP(6,1.7)+100ugDS (Run 10) |
| 6 | -21.63 | 1.83 | VT |
| 7 | -18.70 | 1.81 | |
| 8 | -25.67 | 2.50 | ,DNA=20ug/ml, 25 mM HEPES, pH 8.0 |
| 9 | -22.83 | 2.45 | |
| 10 | -21.59 | 2.07 | |
| Mean | -17.26 | 2.01 | |
| Std. Error | 1.99 | 0.08 | |

| Run | Zeta Potential (mV) | Half Width (mV) | |
|------------|---------------------|-----------------|-----------------------------------|
| 1 | -19.49 | 1.61 | |
| 2 | -30.43 | 3.32 | |
| 3 | -21.66 | 1.68 | |
| 4 | -20.73 | 1.63 | |
| 5 | -19.74 | 1.83 | PLL/DTBP(6,1.7)+150ugDS (Run 10) |
| 6 | -21.84 | 3.94 | VT |
| 7 | -20.72 | 1.70 | |
| 8 | -30.38 | 2.06 | ,DNA=20ug/ml, 25 mM HEPES, pH 8.0 |
| 9 | -16.76 | 2.26 | |
| 10 | -22.71 | 1.92 | |
| Mean | -22.45 | 2.20 | |
| Std. Error | 1.42 | 0.25 | |

| Run | Zeta Potential (mV) | Half Width (mV) | |
|------------|---------------------|-----------------|-----------------------------------|
| 1 | -19.39 | 3.39 | |
| 2 | -23.80 | 2.03 | |
| 3 | -15.61 | 1.90 | |
| 4 | -19.76 | 2.17 | |
| 5 | -17.92 | 2.76 | PLL/DTBP(6,1.7)+200ugDS (Run 10) |
| 6 | -17.77 | 1.71 | VT |
| 7 | -22.13 | 4.28 | |
| 8 | -26.06 | 3.88 | ,DNA=20ug/ml, 25 mM HEPES, pH 8.0 |
| 9 | -18.99 | 1.92 | |
| 10 | -17.95 | 1.99 | |
| Mean | -19.84 | 2.60 | |
| Std. Error | 0.93 | 0.29 | |

| Run | Zeta Potential (mV) | Half Width (mV) | |
|------------|---------------------|-----------------|-----------------------------------|
| 1 | -17.23 | 2.37 | |
| 2 | -8.34 | 1.96 | |
| 3 | -13.48 | 4.20 | |
| 4 | -23.76 | 1.84 | PLL/DTBP(6,1.7)+500ugDS (Run 10) |
| 5 | -18.77 | 1.89 | VT |
| 6 | -16.59 | 4.34 | |
| 7 | -23.00 | 1.95 | ,DNA=20ug/ml, 25 mM HEPES, pH 8.0 |
| 8 | -23.10 | 2.04 | |
| 9 | -22.88 | 2.12 | |
| 10 | -25.96 | 1.84 | |
| Mean | -19.21 | 2.46 | |
| Std. Error | 1.76 | 0.31 | |

12/18/97 Work on recharged ~~cell~~ DNA colloid
(precipitation in salt)

Samples prepared 12/17 (p77) were tested on precipitation
upon addition of NaCl up to 150 mM
Was done with one sample 150 µg DS (close to neutrality point)

I_{50} (700/700)

No.

FI

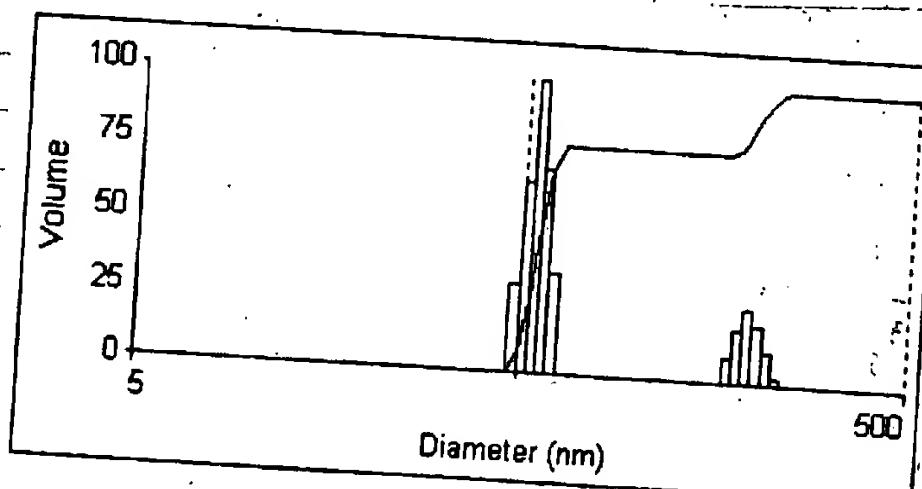
Conc.

| | | | | |
|----|--------------------|------------------|----------|--------|
| 1 | 396.680 | -17246 | coupl | 150±25 |
| 2 | 771.010 | -33522 | cycl | -" |
| 3 | 356.424 | -15496 | alt cent | -" |
| 4 | 484.668 | -21072 | alt cent | -" |
| 5 | 640.237 | -27836 | + salt | |
| 6 | 667.412 | -29017 | + salt | |
| 7 | 618.884 | 26812 | | |
| 8 | 681.295 | 29821 | | |
| 9 | 360.949 | -15693 | alt cent | |
| 10 | 400.766 | -17424 | alt cent | |

There is some

aggregates formed
after each step

but significant
amounts of particles
stays in solution
after addition of salt
and centrifugation



Caged sample

produced
significant intersol.
(≈ 1.2 M cps) after
centrifugation

DNA/PIL (1:6) caged stabilized w 150 µg DS
in 150 mM NaCl after centrifugation

12/23/97 Recharging the colloid with pMAA (not caged)

In standard settings complexes were formed

at 200 µg/ml in 25 mM PEPES pH 8, $\text{PIL/DNA} = 6.1$, $V = 0.5 \text{ ml} / 25 \text{ g}$

→ when pMAA was add

then each 0.5 ml was diluted to 1.5 ml with
the same buffer

I_{50} , TSD, and z potential were measured

0 - 500 μ g pMAA was added to each 25 μ g DNA sample

No. FI Conc.

11 169.143 -7354.0

No. FI Conc.

1 177.670 -7724.8
2 695.225 -30227
3 995.999 -43304
4 320.682 -13942
5 603.757 -26250
6 316.927 -13779
7 456.850 -19863
8 305.441 -13280

No. FI Conc.

1 735.620 -31983
2 68.286 -2969.0
3 64.389 -2799.5
4 580.993 -25260
5 708.999 -30826
6 698.460 -30367
7 744.805 -32382
8 741.753 -32250
9 766.905 -33343
10 45.911 -1996.1

No. FI Conc.

1 125.019 -5435.6
2 339.144 -14745
3 1001.452 -43541
4 964.944 -41954
5 644.407 -28017
6 634.971 -27607

No. FI Conc.

1 393.592 -17112
2 54.019 -2348.7
3 45.936 -1997.2
4 47.624 -2070.6
5 359.647 -15636
6 225.945 -9823.7
7 206.946 -8997.7

^{pMAA}
150 (500/600)

^{pMAA}
mtDNA

690 0
23 25
19 50
535 100
663 200
653 300
699 400
696 500

721 F₀
25 I₅₀

0 DNA 355

0 16

25 7

50 9

100 321

200 187

300 168 47%

38 - F₀

12/30/97 Recharging the DNA / PLL colloid (uncaged)

repetition of experiments from previous page

TOTO

No. FI Conc.

pMMA

| No. | FI | Conc. | F _o | F _v | 881 | 100 |
|-----|---------|---------|----------------|----------------|-------|-----|
| 1 | 13.291 | -577.87 | | | | |
| 2 | 894.401 | -38886 | 0 | 81 | 9.2 | |
| 3 | 94.502 | -4108.8 | 25 | 326 | 37.0 | |
| 4 | 339.541 | -14762 | 50 | 831 | 94.3 | |
| 5 | 844.788 | -36729 | 100 | 888 | 100.7 | |
| 6 | 901.778 | -39207 | 200 | 948 | 104.2 | |
| 7 | 931.606 | -40504 | 300 | 948 | 107.6 | |
| 8 | 961.974 | -41824 | 200 | 965 | 109.5 | |
| 9 | 978.774 | -42555 | 200 | | | |

No. FI Conc.

pMMA - DS

| No. | FI | Conc. |
|-----|--------|---------|
| 1 | 14.718 | -639.91 |
| 2 | 12.247 | -532.48 |
| 3 | 11.329 | -492.57 |
| 4 | 12.886 | -560.26 |
| 5 | 12.353 | -537.09 |
| 6 | 12.194 | -530.17 |
| 7 | 12.591 | -547.43 |

No. FI Conc.

DS

| No. | FI | Conc. | F _o | F _v | 856 | 100 |
|-----|---------|---------|----------------|----------------|------|-----|
| 1 | 29.793 | -1295.3 | | | | |
| 2 | 868.746 | -37771 | 25 | 74 | 8.6 | |
| 3 | 86.448 | -3758.6 | 0 | 50 | 5.8 | |
| 4 | 62.691 | -2725.7 | 25 | 146 | 17.0 | |
| 5 | 158.887 | -6908.1 | 50 | 842 | 98.4 | |
| 6 | 854.383 | -37147 | 100 | 421 | 49.2 | |
| 7 | 433.794 | -18860 | 200 | 359 | 41.9 | |
| 8 | 371.326 | -16144 | 300 | 333 | 38.9 | |
| 9 | 345.736 | -15032 | 500 | | | |

No. FI Conc.

DS - DNA

| No. | FI | Conc. |
|-----|--------|---------|
| 1 | 15.943 | -693.17 |
| 2 | 12.170 | -529.13 |
| 3 | 11.950 | -519.57 |
| 4 | 12.479 | -542.57 |
| 5 | 12.135 | -527.61 |
| 6 | 14.364 | -624.52 |
| 7 | 12.913 | -561.43 |

Conditions are the same
as in p. 80.

TOTO signals from

pMMA alone and

DS alone were measured

DS polyanions did not
change TOTO signals

from DNA.

Igo (100/100)

FI Conc. μ g/ml

| | | | | |
|---|----------|---------|---|-----|
| 1 | 40.148 | -6093.4 | + | 0 |
| 2 | 327.189 | -14225 | + | 25 |
| 3 | 1008.335 | -43840 | + | 50 |
| 4 | 753.784 | -32773 | - | 100 |
| 5 | 559.717 | -24335 | - | |
| 6 | 408.500 | -17760 | - | |
| 7 | 332.728 | -14466 | - | |

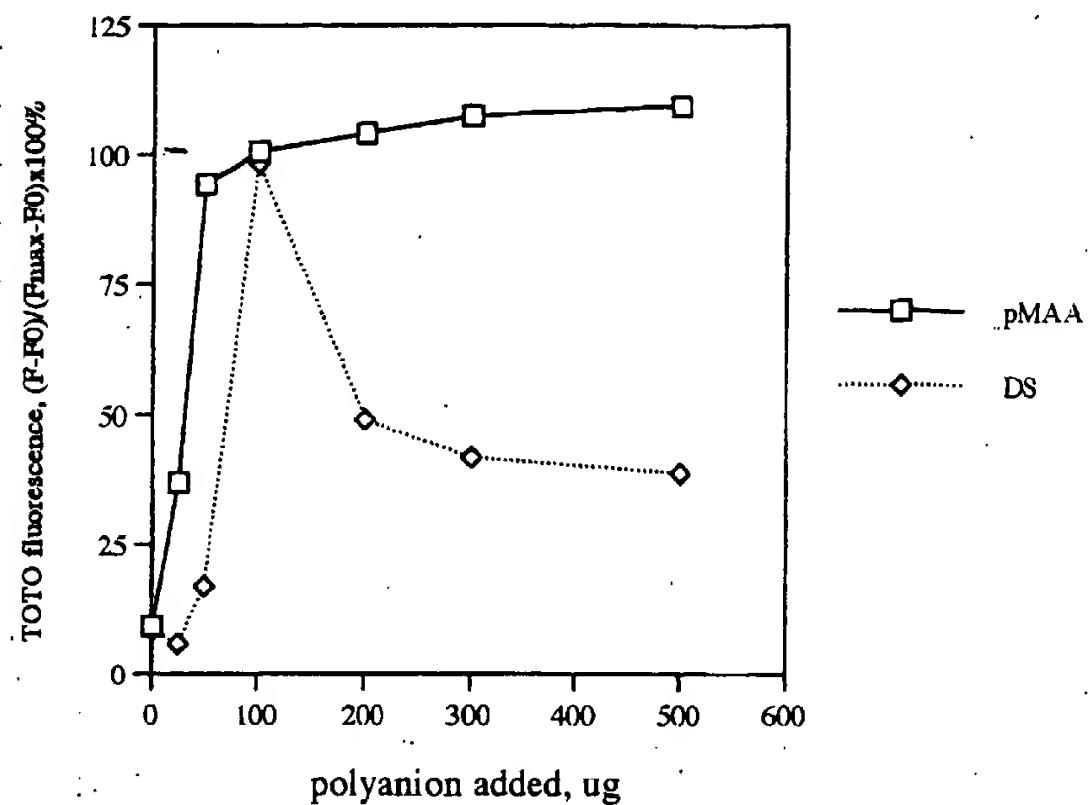
DS

No. FI Conc.

| | | | |
|---|----------|---------|-------|
| 1 | 337.505 | -14674 | + |
| 2 | 1008.335 | -43840 | + |
| 3 | 1008.335 | -43840 | - |
| 4 | 503.257 | -21880 | - |
| 5 | 203.894 | -8865.0 | - 100 |
| 6 | 177.915 | -7735.4 | - |
| 7 | 135.729 | -5901.3 | - |

pMMA

Condensation of DNA/PLL(1:6) upon addition of polyanions



1/6/98. Precipitation of DNA/PLL complex after recharging with polyanion.

the complex DNA/PLL (1:6) + 200 ug DS was prepared as in p. 80. 23.3 g/95 g/200 g in 0.5 ml 25 mM HEPES, pH 8.0.

then it was diluted up to 1.5 ml.

0.5 ml of this solution (17 g/ml) was tested for T₅₀

| No. | FI | Conc. |
|-----|---------|--|
| 1 | 173.291 | -7534.4 DNA/PLL (1:6) |
| 2 | 613.903 | -26691 — 11 — + 200g DS |
| 3 | 219.280 | -9533.9 DNA/PLL aff. cent. |
| 4 | 541.387 | -23538 — 11 — 200g DS + aff. cent. |
| 5 | 723.397 | -31452 DNA/PLL (1:6) in salt. |
| 6 | 984.784 | -42816 — 11 — + 200g DS in salt |
| 7 | 56.981 | -2477.4 DNA/PLL in salt aff. cent. |
| 8 | 588.275 | -25577 — 11 — + 200g DS in salt aff. cent. |

15 min

